Troubleshooting Common HPLC Problems

http://www.hplc1.com/shodex/english/dd.htm
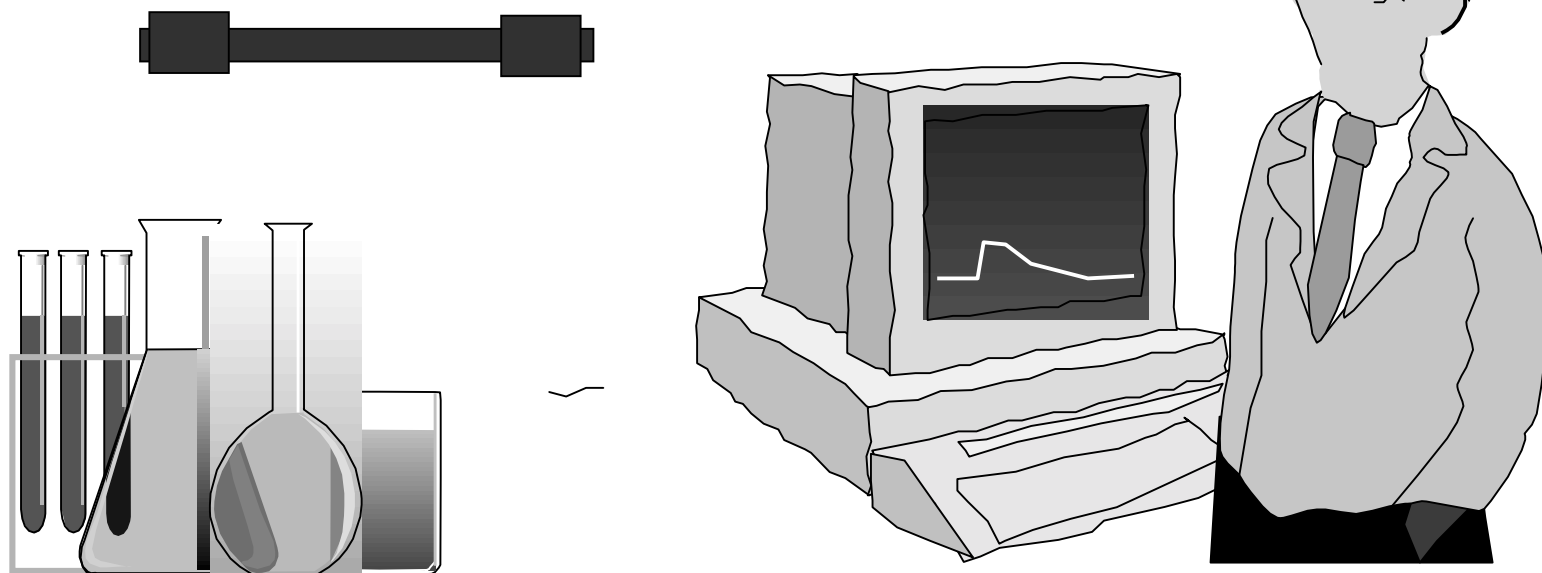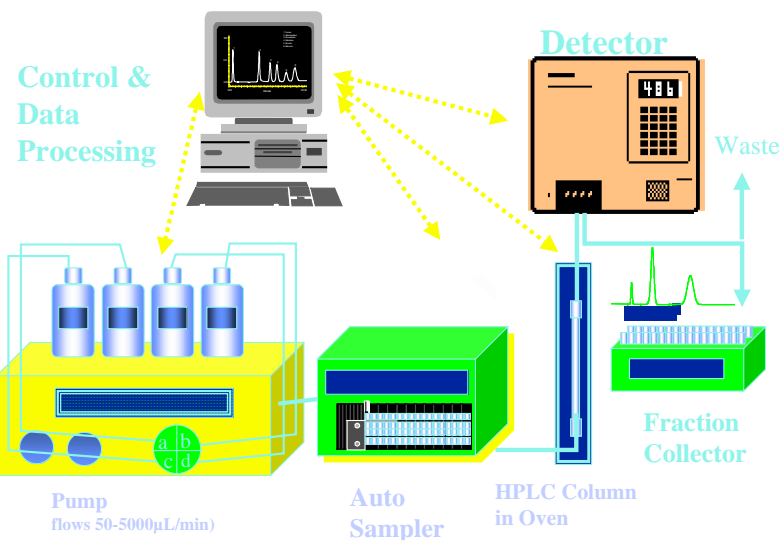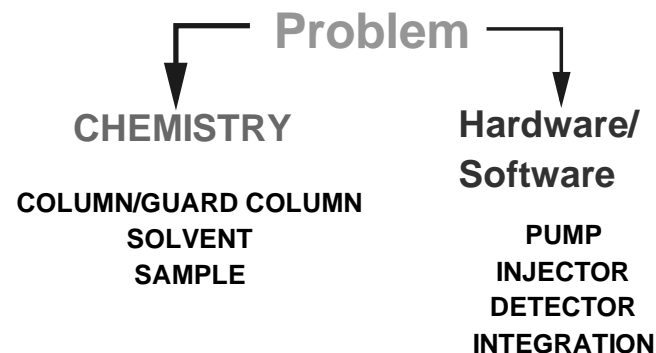
HPLC
Troubleshooting

Performance Monitoring

Use Your Test Method
(Known Performance)

* Monitor at least One Peak in one injection
  - Plate Count (Peak width relative to RT),
  - Peak Asymmetry,
  - Retention Time and/or Retention parameter
  - Relative Retention Time for Critical Pair of Analytes.
  - Peak Response

* Inject Multiple Runs
  - Precision (at least 5 injections)
  - Accuracy (Use Control Samples)

Control & Data Processing

Detector

488

Waste

Fraction Collector

Pump
flows 50-5000μL/min)

Auto Sampler

HPLC Column in Oven

Troubleshooting

Problem

CHEMISTRY	Hardware/ Software
COLUMN/GUARD COLUMN	PUMP
SOLVENT	INJECTOR
SAMPLE	DETECTOR
	INTEGRATION

Dr. Shulamit Levin, Medtechnica

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Performance Monitoring

Use Your Test Method (Known Performance)

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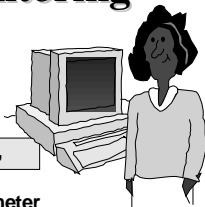   - Precision (at least 5 injections)
    - Accuracy (Use Control Samples)

Plate Count - Efficiency of the Separation

* A "Plate Count" Actually Is a Determination Of Both The Column AND Instruments' Performance

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Performance Monitoring

Column Efficiency:
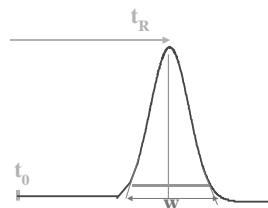N = the number of Theoretical Plates
a = is a constant depending on the Method used
t_r = retention time of peak
W = the peak width (time units) at a given peak height

$$N = a \left(\frac{t_r}{W} \right)^2$$

METHOD	a
Peak Width at Half Height	5.54
Peak Width at 4.4% Peak Height (5 Sigma)	25.0
Tangent	16.0

Performance Monitoring

Band Spreading

* Band Spreading Impacts Chromatographic Performance -- The Greater The Band Spreading, The Poorer The Performance (ie; Resolution)

* Band Spreading Contains Both An Instrument AND A Column Contribution

Extra-Column Band Spreading

The Observed Bandwidth (TOT)

* Sum of the Bandspreading Contributions
- **Column (COL)**
- **Extra-Column (EC) Instrument components**

$$\sigma^2_{TOT} = \sigma^2_{COL} + \sigma^2_{EC}$$

Band Spreading

* Column Contribution

σ^2_{COLUMN} = optimized by choosing the correct column and conditions
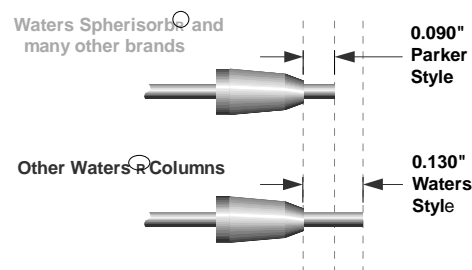
* Instuments Contribution = Extra-Column

$$\sigma^2_{EC} = \sigma^2_{TUBING} + \sigma^2_{CONNECTIONS} + \sigma^2_{INJECTORS} + \sigma^2_{DETECTORS}$$

Performance Monitoring

Extra-Column Band Spreading
**(Instruments' Contribution)**

1. **Injection Volume**
2. **Injector**
3. **Connection Tubing**
   a. **from Injector to Column**
   b. **from Column to Detector**
   c. **Endfittings and Frits**
4. **Detector Volume**

Connectors

Waters Spherisorb® and many other brands — 0.090" Parker Style

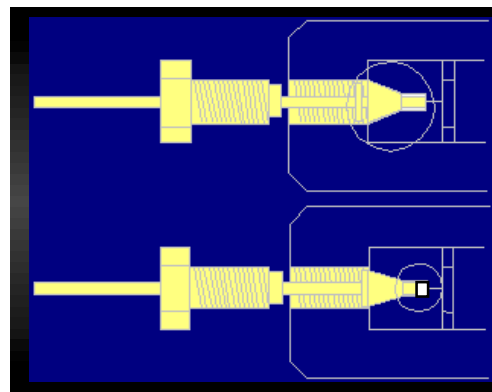Other Waters® Columns — 0.130" Waters Style

Installation and Equilibration

✓ Make sure column inlet connected correctly
✓ Make sure nut and ferrule are seated

Good Seal
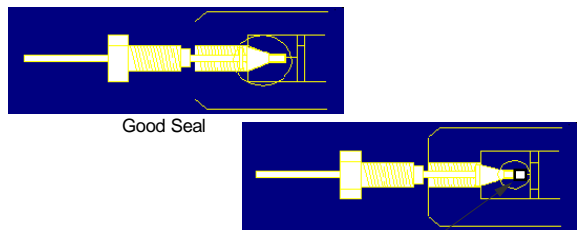
NOTE: column inlet connector not seated properly
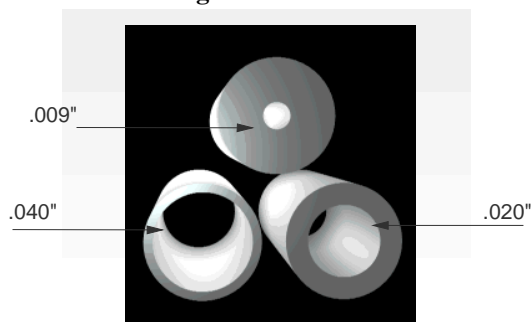
{PEEK Connectors Easier to Use --
THF makes PEEK brittle}

Extra-Column Band Spreading

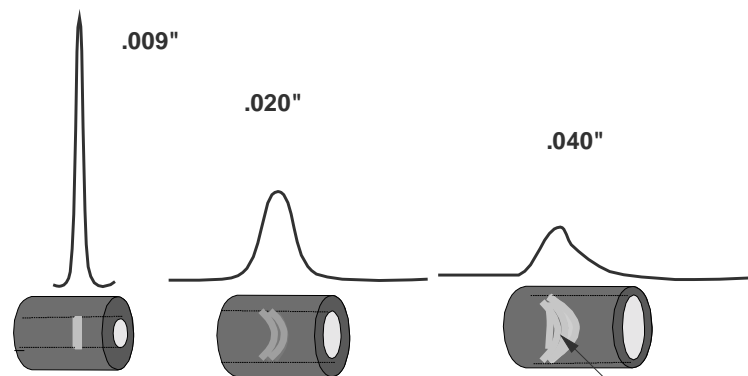**Column Connection Contribution**

Extra-Column Band Spreading

Tubing Contribution

.009"

.040"   .020"

note the differences of the inner diameter of this tubing

Performance Monitoring

Effect of Connecting Tubing on System Bandspreading

.009"

.020"

.040"

Diluted/Distorted Band

sample band dispersion inside tubing

Measuring The Instruments Contribution

* **Perform An Instrument Band Spreading Test**

Performance Monitoring

To perform a measurement:
  - **disconnect column from system**
  - **connect injector directly to detector**

Parameter	Setting
Flow Rate	1.0 mL/min
Chart Speed	20 cm/min
Detector Sensitivity	0.5 - 1.0 AUFS
Time Constant	0.2 seconds or less

dilute test mixture 1 to 10 in mobile phase
inject 2 to 5 μl of this solution

Performance Monitoring

Using 5 sigma efficiency method, measure the peak width
  at 4.4% of peak height
Convert to microliters using the following equation:

$$\left(\frac{2cm}{PW} \right)\left(\frac{1min}{20\ cm} \right)\left(\frac{1\ mL}{min.} \right)\left(\frac{1000\mu L}{mL} \right) = 100\ (\mu L)$$

where:
1min/20cm = chart speed
1 mL/min = flow rate
1000 μL/mL = volume correction factor

Typical LC System should be 100μL +/- 30μL
Microbore System should be no greater than 20μL

Performance Monitoring

Impact of System Band Spread on a Plate Count:

- System with 70μl Band Spread >> 10,000 plates
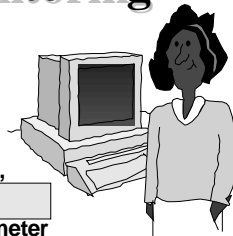
- **System with 130μl Band Spread >> ~8,000 plates**

On the Same Column!

Assumption: <40% loss in resolution at k' = 5 and N= 10,000 and <20% loss in resolution at the preferred value

Performance Monitoring
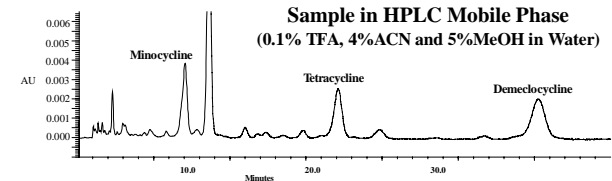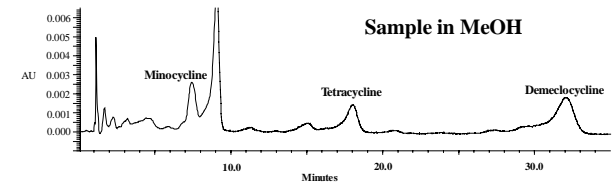
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* Inject Multiple Runs
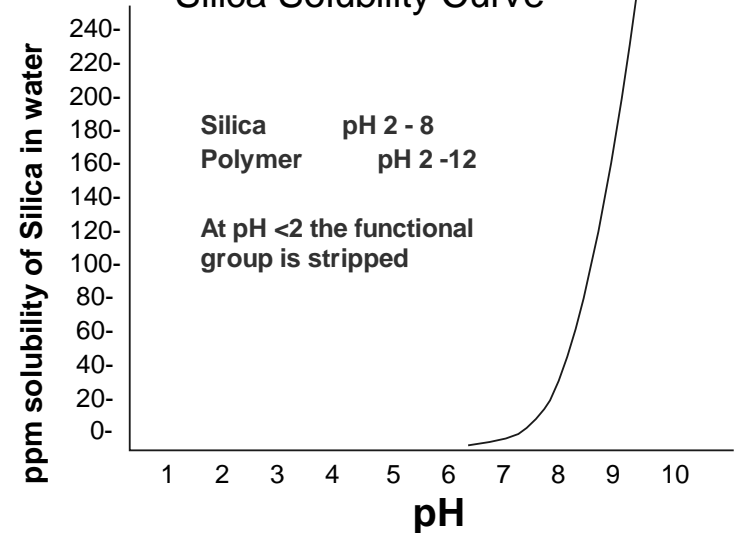 - Precision (at least 5 injections)
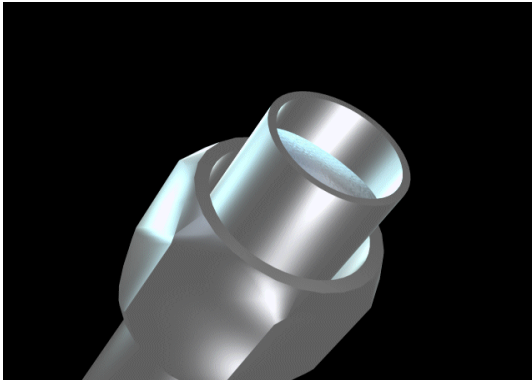 - Accuracy (Use Control Samples)

Incorrect Sample Solvent

Sample in MeOH

Minocycline   Tetracycline   Demeclocycline

10.0   20.0   30.0
Minutes

Sample in HPLC Mobile Phase (0.1% TFA, 4%ACN and 5%MeOH in Water)

Minocycline   Tetracycline   Demeclocycline

10.0   20.0   30.0
Minutes

Column Use

✓ Silicas hydrolyze at high pH

✓ Instability of bonded phase at low pH

✓ Elevated temperatures decrease column lifetime

✓ C18 approximately 1000 times more stable than CN

Silica Solubility Curve

Silica   pH 2 - 8
Polymer   pH 2 -12

At pH <2 the functional group is stripped

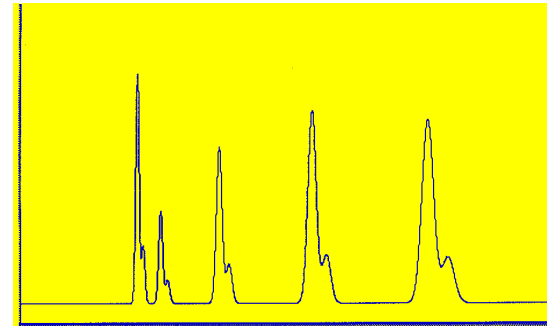ppm solubility of Silica in water

pH

Column Collapse
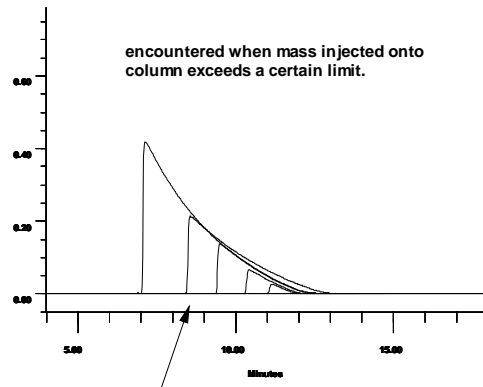

voided column

Column Collapse

voids - high back pressure,
distorted and/or double peaks

Mass Overload

encountered when mass injected onto
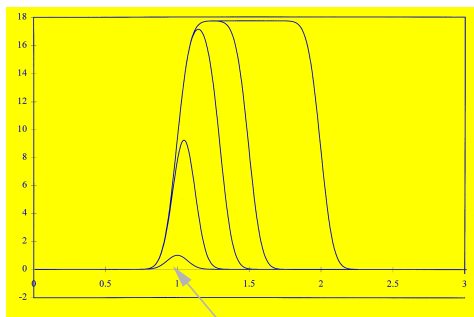column exceeds a certain limit.

Lift-off Point Moves Earlier
Retention times are shorter

Column/Volume Overload

500 μL   300 μL   100 μL   10 μL
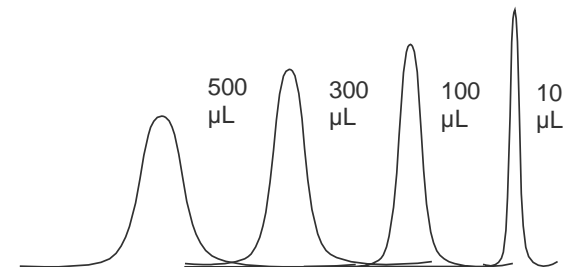
EFFECT OF INJECTION VOLUME
ON PEAK DISTORTION

Volume Overload

Lift-off Point Remains Constant
Retention times are longer

Contaminated In-Line Filter
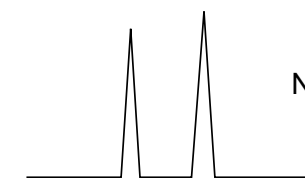
New frit = 800 psi
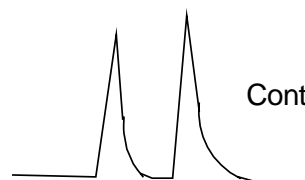
Contaminated frit = 2500 psi

Extra Column Effects

Isocratic LC - Time Constant Differences
(Detector setting)

left is 0.1 secs   right is 10 secs
note the noisy baseline on left chromatogram

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  - Relative Retention Time for Critical Pair of Analytes.
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Retention Time Problems

- Reproducibility
  - ► Solvent Composition
  - ► Temperature
  - ► pH-Control
  - ► Ion Pairing

- Drifting Retention
  - ► Equilibration
  - ► Stationary Phase Stability
  - ► Column Contamination
  - ► Hydrophobic Collapse

Solvent Composition

- Clearly specify HOW the Mobile Phase is to be prepared

60/40
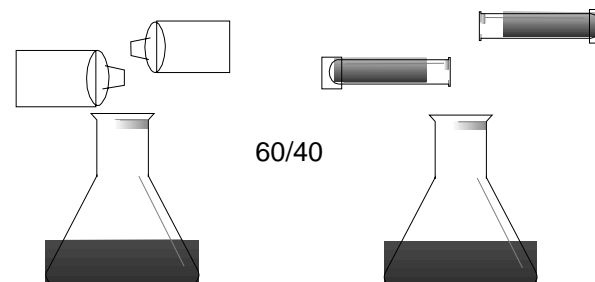
pH Reminder: Measure pH *Before* the organic is added

Temperature Control

Retention Time Reproducibility

Non-Column Influences:

pH

- Neutrals: No Influence

- Acids: Reduced Retention with Increasing pH

- Bases: Increased Retention with Increasing pH

- 10% Change in Retention per 0.1 pH Units

Reversed-Phase Retention Behavior of Acidic Compounds Relative to Changes ± 1 pH Unit from pK$_a$

- 1 pH unit = 91% un-ionized

Un-ionized Acid

Small Change in pH = Large change in k (potential reproducibility problems)

pK$_a$

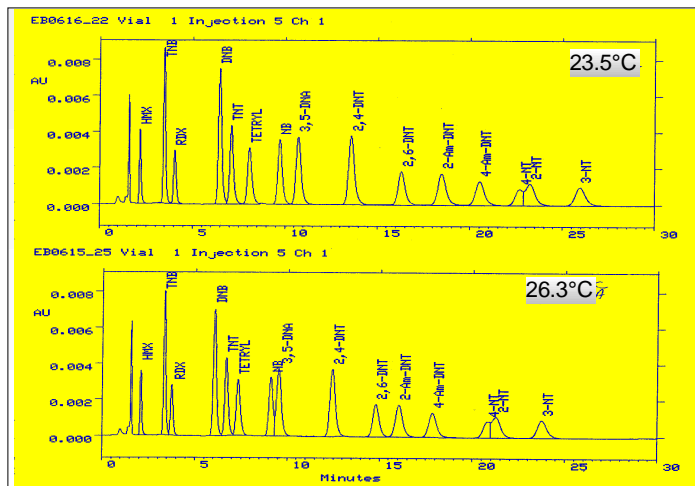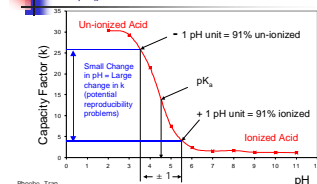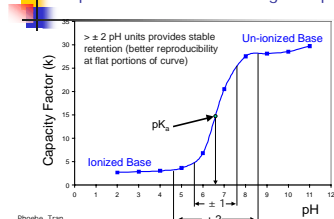+ 1 pH unit = 91% ionized

Ionized Acid

Phoebe, Tran
© Waters Corporation 2000
(101300)

Reversed-Phase Retention Behavior of Basic Compounds Relative to Changes in pH

> ± 2 pH units provides stable retention (better reproducibility at flat portions of curve)

Un-ionized Base

pK$_a$

Ionized Base

Phoebe, Tran
© Waters Corporation 2000
(101300)

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pH Control — AZT: Robustness Testing

6% Methanol, 6% THF

Changing Retention Times

Retention times getting shorter after each injection?

Sample analytes can adhere to and cover active functional group sites making a shorter column

Before injection

15 cm

After injection

Covered functional groups

12 cm
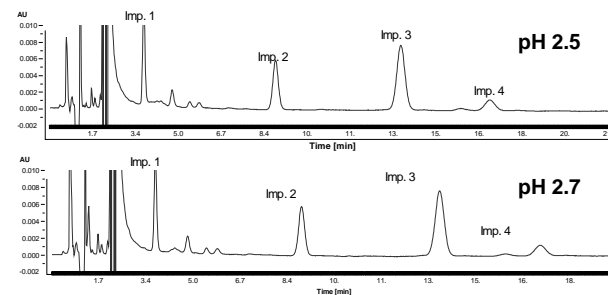
COLUMN REGENERATION

REVERSE PHASE

1. Wash with unbuffered mobile phase
2. Wash with 100% water
3. Wash with methanol (or ACN)
4. Wash with THF or IPA
5. Wash with methylene chloride
6. Wash with N-Heptane
7. Wash with methylene chloride
8. Wash with methanol (or ACN)
9. Wash with water
10. Return to solvent

Installation and Equilibration

✓ **Purge column with 10 column volumes of mobile phase to be used in analysis**
  (>>> 4.6x150mm = 25mL)

✓ **Reversed-Phase (C18 etc.) columns equilibrate quicker than Normal Phase columns**
  ➡ **(magnitude of ten)**

✓ **Normal phase columns (silica or alumina) may take several DAYS at flow rates of 1.0 ml/min**

Installation and Equilibration

Internal Diameter (mm)	Length (mm)	Column Volume (mL)
2.0	150	.47
2.0	300	.94
3.9	50	.6
3.9	75	.9
3.9	100	1.2
3.9	150	1.8
3.9	300	3.6
4.6	150	2.5
4.6	250	4.2
5	100	2.0
8	100	5.0
7.8	300	4.3
19	150	43
25	100	49
30	300	212
40	100	125
47	300	520
50	300	589

Solvent Viscosities

Solvent	Viscosity [cP] at 20° C
Acetone	0.32
Acetonitrile	0.37
Cyclohexanone	0.98
Di-isopropylether	0.37
Diethyl ether	0.23
Dimethyl acetamide	2.1
Dimethyl formamide	0.92
Dimethyl sulfoxide	2.2
Dioxane	1.54
Ethanol	1.2
Ethyl acetate	0.45
Hexafluoroisopropanol	1.0
iso-Propanol	2.5
Isooctane	0.5
Methanol	0.6

Remember: Some mixtures are more viscous than either pure solvent -- 50/50 MeOH/H2O is almost 2x

Solvent Viscosities

Solvent	Viscosity [cP] at 20° C
Methyl acetate	0.37
Methylene chloride	0.44
Methylethyl ketone	0.4
n-Heptane	0.42
n-Hexane	0.33
N-Methyl pyrrolidone	1.67 (25? C)
n-Pentane	0.235
n-Propanol	2.3
o-Dichlorobenzene	1.41
Tetrahydrofuran	0.46
Toluene	0.59
1.2.4-Trichlorobenzene	1.89 (25? C)
Water	1.0
m-Xylene	0.62
o-Xylene	0.81

Remember: Some mixtures are more viscous than either pure solvent -- 50/50 MeOH/H2O is almost 2x

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Column Protection

Major cause of column deterioration is contamination.
Use of guard columns may increase column life-time
to > 10,000 analyses

30mm guard column

column coupler
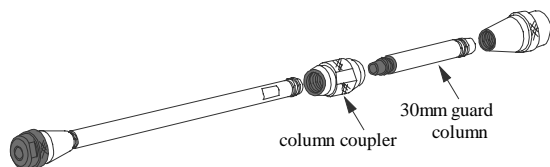
Column Protection

1. **Guard column should be regarded as a cost-effective sacrifice to extend analytical column life-time**

2. **Should contain IDENTICAL packing material as the analytical column**
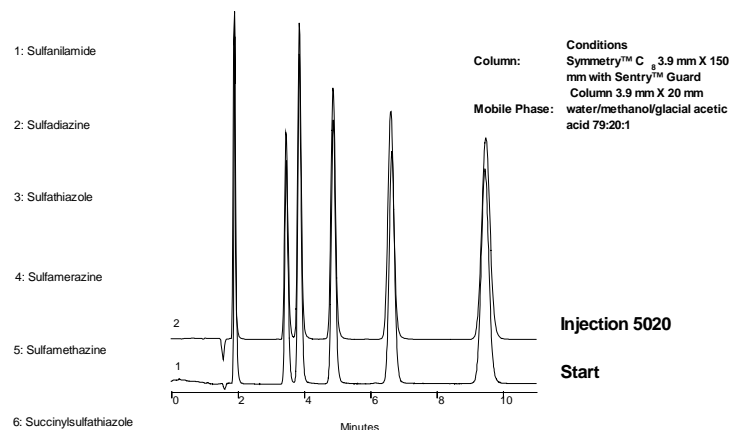   **e.g. using a different C18, with different retention properties could actually destroy the separation**

Well designed, well packed guard column will actually IMPROVE the analytical separation efficiency

Column Protection
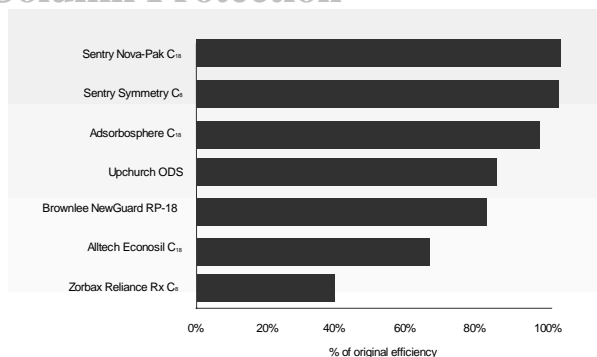
Effect of guard column on HPLC columns efficiencies
Analytical column Nova-Pak C18 (150 x 3.9mm or 4.6mm) except Zorbax Rx C8 (150 x 4.6mm)
Sample was 0.5μL injection acenapthene (2.9 mg/mL) and acetone (34 μL/mL) in ACN/Water

Column Protection

1: Sulfanilamide

2: Sulfadiazine

3: Sulfathiazole

4: Sulfamerazine

5: Sulfamethazine

6: Succinylsulfathiazole

Conditions
Column: Symmetry™ C$_8$ 3.9 mm X 150 mm with Sentry™ Guard Column 3.9 mm X 20 mm
Mobile Phase: water/methanol/glacial acetic acid 79:20:1

Injection 5020

Start

Minutes

Chromatogram of Life-time Test

* Guard Column Changed Every 500 Injections

Column Protection

A. Initial injection on Symmetry C8 Sentry guard column

A

B. After 550 injections on same Sentry guard column

B

C. New Sentry Guard column for injection 551 on analytical column

C

Minutes
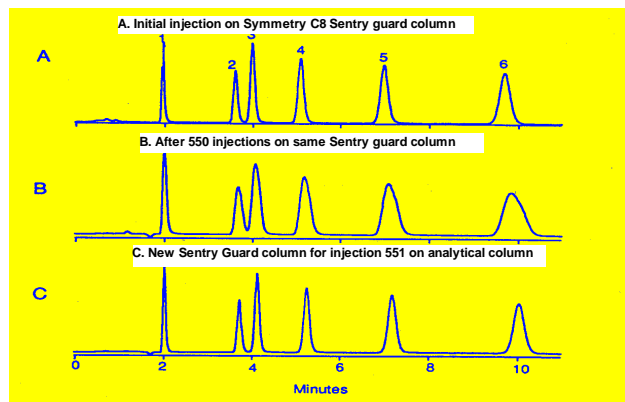
Extension of column lifetime with Guard Column using a mixture of sulfa drugs as the sample

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- **Peak Asymmetry,**
- **Retention Time and/or Retention parameter**
- **Relative Retention Time for Critical Pair of Analytes.**
- **Peak Response**

* Inject Multiple Runs
- **Precision (at least 5 injections)**
- **Accuracy (Use Control Samples)**

Variable Reported Concentrations Problems with Peak Response

■ Linearity Test of Concentrations

- Check Injector (Use Standards)
  * Multiple Injections - Same Vial -- Syringe Problem
    or If Only 1st Injection Low -- Septa Problem
  * Different Vials -- Evaporation -- Degradation
  * Injection Volume Test (Weight before and after injection)

- **Integration Software**
  * Electronic Peak Generator
  * Poor Peak Shape

- **Detector**
  * Cell Problem
  * Lamp Failing

RSD < 5-15%

0.010

AU 0.005

0.000

51.8 52.0 52.2 52.4 52.6 52.8
Minutes
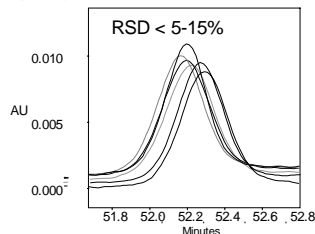
Troubleshooting your UV detector

0.01

S R AUFS Offset
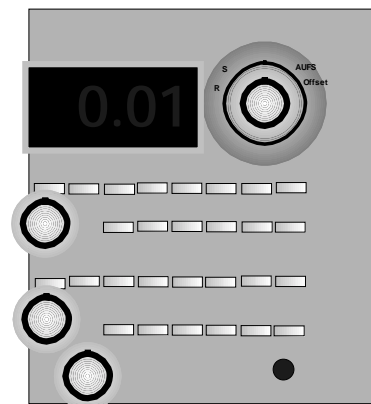
► **Reference Energy**
► **Sample Energy**
► **Absorbance**
► **Offset**

Unusual Phenomena

- ► Extraneous Peaks
- ► Problems with Baseline
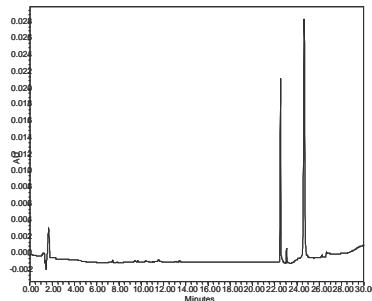
Isocratic LC - Extra Peak - Sharp - Contaminant
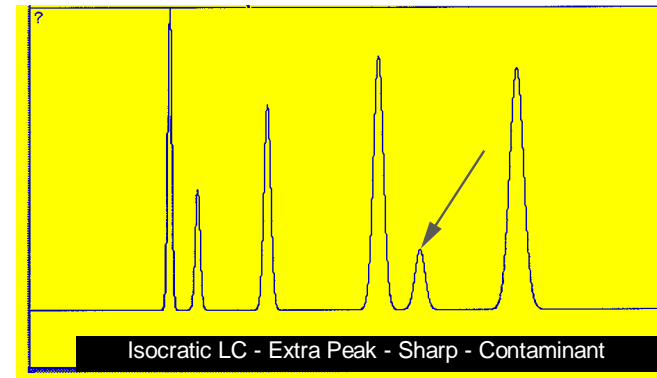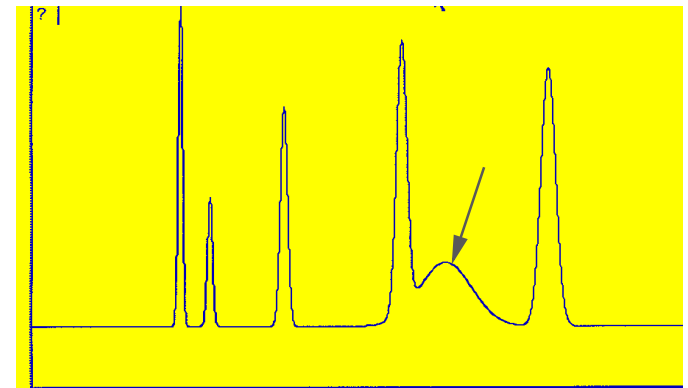
Extraneous Peaks
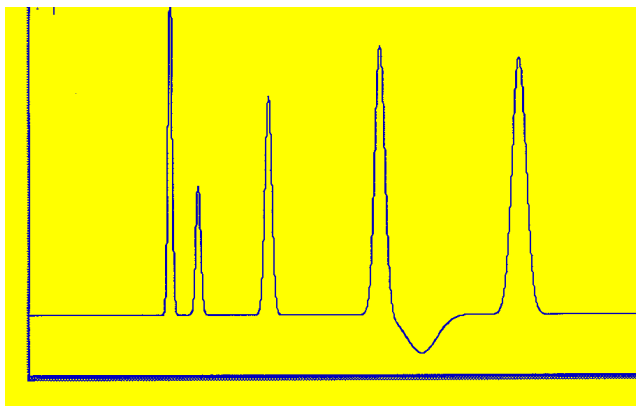
Sample
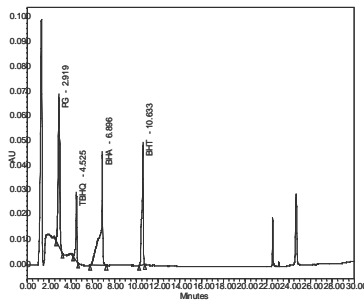
Blank

Extraneous Peaks

Isocratic LC - Broad -Peak from Previous Injection or Injector Contamination

Isocratic LC - Negative Peak
often occurs in Ion-Pairing -- Sample Solvent

Installation and Equilibration

✓ Connect Column Inlet

✓ Purge Column at Low Flow Rate To Waste --
Then Connect to Detector

(begin flow of analytical columns at 0.1 ml/min
increase by 0.2 ml/min increments every 30 seconds until
final analytical flow rate is reached)

mobile phase flow direction

HPLC Pump   Manual   Guard   HPLC Column
             Injector   Column

Detector

Degas Solvents

Vacuum

Ultrasonic bath   Time = 1 minute
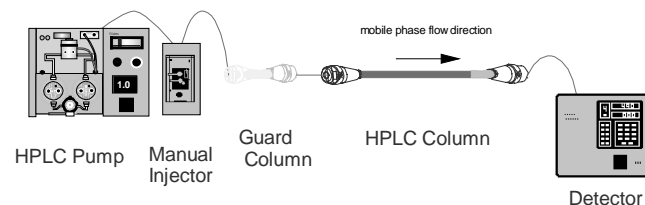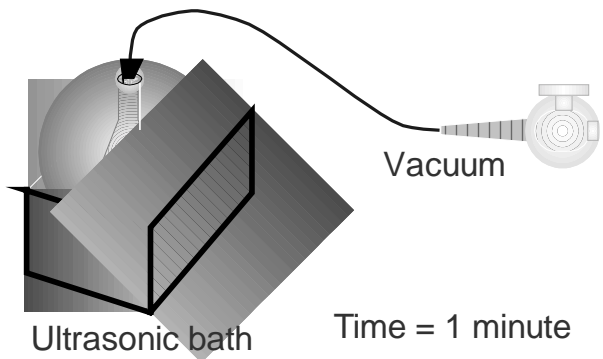
Solvent Degassing Precautions

1. **Degas solvents prior to adding modifiers**

2. **Helium sparge is good, as long as solvent doesn't change due to volatility of solvents and/or additives**

3. **Solvents should be degassed daily**

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BASELINE TROUBLESHOOTING

Noisy baseline

Synchronous noise

Asynchronous noise

Drift

Cyclic

Spikes

No peaks

Positive & negative peaks

NOISY BASELINE

INSTRUMENTAL

WEAK DETECTOR LAMP
Replace lamp
LEAKS
Stop leaks. Replace fittings
DETECTOR CELL DIRTY
Flush with 6N nitric acid
GAS IN MOBILE PHASE
Degas solvent
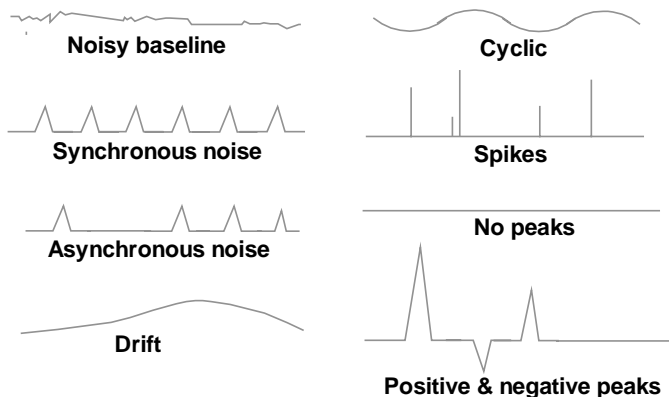GAS BUBBLE IN DETECTOR CELL
Put .009" tubing after detector (not RI!)
ELECTRONIC NOISE
Remove source. Shield cables. Clean contacts
SENSITIVITY TOO HIGH
Lower sensitivity. Adjust gain

CHEMICAL

TRASH ELUTING OFF COLUMN
Flush column with strong solvent

SYNCHRONOUS NOISE

ALMOST ALWAYS CAUSED BY THE PUMP

Air in pump head - Prime pump and degas solvent

Check valve problem - Rebuild or replace

Broken plunger - Replace (blame it on someone else)

Mixing problem - Increase system volume

Electrical noise - Change circuits, remove source

ASYNCHRONOUS NOISE

BUBBLES
Degas mobile phase
GAS CAUGHT IN DETECTOR
Degas mobile phase. Put
backpressure on cell.
LEAKS
Fix leaks, replace fittings
MIXING PROBLEMS
Increase system volume
PLUGGED LINES
Remove plug, flush system
ELECTRICAL PROBLEMS
Remove source, change circuits

BASELINE DRIFT

INSTRUMENTAL

GRADIENT - SOLVENT B ABSORBS MORE THAN SOLVENT A
> Try a new mobile phase, use baseline subtraction

SOLVENT CHANGING (GAS ABSORPTION, EVAPORATION
> Helium sparge, enclose solvents

SOLVENT LEAKS
> Tighten, replace fittings

THERMAL EFFECTS (ESPECIALLY RI, CONDUCTIVITY, ECD)
> Cell temperature regulation

BACKPRESSURE CHANGES
> Filter solvents and samples. Sample too viscuous

SIPHONING (RI, CONDUCTIVITY, ECD)
> Increase system volume

MIXING PROBLEMS

CHEMICAL

COMPOUNDS ELUTING OFF COLUMN
> Run strong solvent until baseline is stable

SOLVENTS IN GRADIENT ARE NOT PURE
> **Change the solvent batch or manufacturer.**
> **Check if the solvents are grandient grade.**

CYCLIC BASELINE

TEMPERATURE FLUCTUATIONS
> Thermally insulate. Move away from ventilation.
> Increase cell temperature.

MIXING PROBLEMS
> Increase system volume

GAS IN MOBILE PHASE
> Degas solvents

ELECTRICAL PROBLEMS
> Change circuits, remove source

ERRATIC PUMP
> Repair pump

PLUG
> Remove obstruction, flush system

SPIKES

BUBBLES
> Degas solvent

POOR ELECTRICAL CONNECTION, LOOSE WIRING
> Clean and tighten detector leads, check wiring, replace spade lugs.

LAMP RELAY TRYING TO FIRE A DEAD LAMP
> Replace lamp

ELECTRICAL NOISE
> Change circuits, remove source
> Common sources include switching valves, compressors, muffle furnaces, fraction collectors, power conditioners, lighting, poor power source.

NO PEAKS

INSTRUMENTAL

- **Injector not making injections**
- **Pump not pumping**
- **Dead detector**
- **Integrator/recorder not wired correctly**
- **Gain setting too low**
- **Leaks**

WHAT TO DO:
Inject acetone solution to make a peak

CHEMICAL

- **Column retaining all compounds**
- **Bad or wrong mobile phase**
- **Bad or wrong standard or sample**
- **Wrong guard column**

WHAT TO DO:
Remove column and inject acetone solution to make a peak

NEGATIVE & POSITIVE PEAKS

INSTRUMENTAL

Air bubbles passing through cell
Degas mobile phase

You're using an RI detector
May be normal since peak direction is a function of
refractive index differential from mobile phase

All peaks negative - polarity wrong
Reverse leads or change detector polarity

All peaks negative - You're using indirect UV
Change polarities or reverse leads

CHEMICAL

Some eluting compounds absorb less than solvent
Use a different or cleaner solvent

Basic assumptions

1. The HPLC is plugged in and turned on
2. Solvent is in the reservoir
3. The pumps are primed and in good working order
4. The HPLC is plumbed and wired correctly
5. The detector has a good lamp in it
6. The solvent bottle doesn't have a vacuum on it
7. You're not using acetone for solvent at 195 nm
8. You're not injecting rocks
9. You're not doing a water to hexane gradient
10. Your're not trying to detect sugars at 254 nm
11. You're not mixing MEOH and water without degassing
12. You're not sparging with nitrogen or air
13. You're not running water through a silica column
14. Solvent pH is not 13 on a silica base column
15. You're not running a 1M NaCl to 100% ACN gradient
16. You're not doing gradients with an RI detector
17. You're RI is not under the air conditioner vent
18. No buffer stalagtites on your pump heads
19. HCl vapors are not blowing onto your HPLC
20. You're having a wonderful time!

Strange things can happen!

Radio transmitters can cause baseline noise

Contaminated helium bottles and lines can cause noise

System components can get coated with impurities

Solvent vendors can misname solvent bottles

Some filters can introduce particulates

Things not to do:

* **Plug the outlet of your RI detector**
* **Flush your system with methanol after running buffer**
* **Inject samples that may precipitate in the eluent**
* **Run long durations with HCl on your stainless steel HPLC**
* **Filter organic solvents through aqueous filters**
* **Spill buffers onto HPLC electronics**
* **Try to change the column frits while it still has pressure in it**
* **Store THF on the shelf, uncapped, for weeks**
* **Pump cyclohexane above 2000 psi**
* **Tightly seal your mobile phase container**
* **Cut tubing with a wire cutter**